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GB A 2085447

GB A 2028342

GB 1589738

GB 1518207

GB 1510926

GB 1510925

EP A1 0077124

WO A1 8200641

WO A1 8002559

(58) Field of search  
C3H

(54) A method capable of determining cathepsin B in the presence of other proteolytic enzymes and compounds useful therefor

(57) A method of selectively assaying for the activity of cathepsin B in mammalian fluids and tissues which may also contain trypsin and trypsin-like enzymes using novel substrates. The substrates are of the general formula: Z - R<sub>2</sub> - R<sub>1</sub> - X wherein X is an indicator moiety cleavable by cathepsin B, R<sub>1</sub> is an amino acid group which is not positively charged, R<sub>2</sub> is a hydrophobic amino group, and Z is an amino blocking group.

GB 2 140 423 A

## SPECIFICATION

A method capable of determining cathepsin B in the presence of other proteolytic enzymes, and compounds useful therefor

5 This invention relates to a method capable of measuring cathepsin B activity in the presence of other proteolytic enzymes such as trypsin. This invention further relates to novel substrates which are highly specific to cathepsin B. The method of this invention is particularly useful for the detection of abnormal levels of cathepsin B activity in body fluids, such as human blood, and in body tissues.

10 Proteinases are enzymes that digest proteins and polypeptide chains. These enzymes are highly selective to cleavage sites of the polypeptide substrates. This enzyme activity is precisely controlled in a normal biological system. A breakdown in this control can lead to serious biological consequences. Numerous disease states have been attributed to such breakdowns or disturbances in the activity of proteinases (as discussed in A. J. Barrett, ed. (1979) *Proteinases in Mammalian Cells and Tissues*, North-Holland, New York; 15 A. J. Barrett and J. K. McDonald (1980) *Mammalian Proteases: A Glossary and Bibliography*, Vol. 1, Academic Press, New York).

Cathepsin B is a normal lysosomal cysteine (thiol) proteinase. Recently it has been shown (A. J. Barrett and J. K. McDonald, *op. cit.*; M. Sandler, ed. (1980) *Enzyme Inhibitors as Drugs*, University Park Press, Baltimore) that an apparent breakdown in the control of cathepsin B activity may lead to a variety of disease states.

20 Several diseases associated with collagen and structural glycoprotein breakdown have been linked to increased cathepsin B activity. In particular, a correlation between cathepsin B activity and metastatic disease has been suggested by several investigations, including the elevation of cathepsin B or cathepsin B-like activity in the blood of patients with metastatic disease [as discussed in R. J. Pietras, et al. (1978) *Obstet. Gynecol.* 52; 321-327; R. J. Pietras, et al (1979) *Gynecol. Oncol.* 7; 1-17.] Accordingly, a method for 25 the accurate and selective assay of cathepsin B activity in mammalian body fluids would be a useful diagnostic aid, such as detecting or monitoring.

The assays of various enzyme activities are routine procedures in the clinical laboratory. Typically such assays include contacting the enzyme-containing sample with a synthetic substrate (defined as that substance or compound acted upon by the enzyme) that is selectively cleaved by that enzyme into products 30 that change colors:

	substrate	enzyme	
	(no color)	→	products
			(colored)
35	(colored)	or	(no color)

Thus, substrates for cathepsin B would be peptide or peptide-like compounds that upon cleavage allow analysis of the products. Assays that have been used for cathepsin B [as discussed in A. J. Barrett, *op. cit.*; R. J. Pietras, *op. cit.*; A. J. Barrett (1980) *Biochem. J.* 187: 909-912] have included peptide substrates that 40 incorporate one or more arginyl or lysyl amino acids attached to an enzyme-cleavable indicator group. (e.g. Bz-Arg-NA, CBZ-Lys-PNP, CBZ-Arg-Arg-MNA, CBZ-Ala-Arg-Arg-AFC, CBZ-Phe-Arg-AMC, see abbreviations below). While the selective cleavage of synthetic substrates by cathepsin B is not yet well understood, these currently used substrates, by the nature of their positively-charged groups, will also be cleavable by the 45 trypsin-like (serine) proteases. Thus, the assays for cathepsin B using one of these lysyl or arginyl substrates in mammalian body fluids is complicated by the presence of abundant trypsin-like proteases. In particular, it has not been known how to achieve high specificity for and detection of cathepsin B activity in the presence of trypsin and trypsin-like enzymes.

Accordingly, an object of the invention is to provide substrates highly specific for cathepsin B. It is another 50 object of the invention to selectively assay for cathepsin B activity in the presence of other proteolytic enzymes, for example, of trypsin. It is yet another object of the invention to determine cathepsin B activity in mammalian body fluids and tissues. Further objects of the invention will be apparent from the following description.

Thus viewed from one aspect the invention provides a method of selectively assaying for the activity of 55 cathepsin B in a material to be tested which may contain trypsin and trypsin-like enzymes, the steps comprising:

(a) mixing a sample of said material with a substrate of formula I



60 (wherein: 60

X is an indicator moiety released by cleavage of the  $R_1 - X$  bond by cathepsin B and which is detectable upon cleavage;

$R_1$  is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and 65 which is not positively charged within the pH range for the assay; 65

R<sub>2</sub> is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

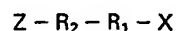
Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the R<sub>1</sub> and R<sub>2</sub> groups) or an acid salt thereof,

5 said mixing being carried out in an aqueous medium having a pH within the range at which cathepsin B is active and using a quantity of said substrate substantially greater than that of cathepsin B and in sufficient concentration for cleaved X-groups to be detectable; and 5

(b) measuring the rate of cleavage of the X-group from said substrate.

Viewed from a further aspect, the invention provides a method of detecting the activity of cathepsin B in a material to be tested which may contain trypsin and trypsin-like enzymes, and which is adapted to selectively assay for cathepsin B, the steps comprising: 10

(a) contacting a sample of said material with a substrate of formula I



(I)

15 (wherein: 15

X is an indicator moiety released by cleavage of the R<sub>1</sub> - X bond by cathepsin B and which is detectable upon cleavage;

R<sub>1</sub> is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and which is not positively charged within the pH range for the detection method; 20

R<sub>2</sub> is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the R<sub>1</sub> and R<sub>2</sub> groups) or an acid salt thereof,

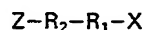
25 said contacting being carried out under conditions at which cathepsin B is active and at concentrations at which said substrate is present in amounts substantially greater than that of cathepsin B; and 25

(b) determining the production of X cleaved from said substrate.

This invention provides a method for testing for cathepsin B activity in sample materials, especially mammalian body fluids or tissues, by measuring the rate at which an indicator group X is released upon

30 cleavage of a synthetic peptide substrate highly selective for cathepsin B. 30

Viewed from a further aspect, the invention provides compounds of formula I



(II)

35 (wherein X is an indicator moiety cleavable from R<sub>1</sub>; 35

R<sub>1</sub> is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group (C<sub>α</sub>) and that is not positively charged (within the pH range of the testing conditions), preferably having a polar side chain, and preferably in which the side chain has from 2 to 8 atoms;

R<sub>2</sub> is a hydrophobic amino acid residue, preferably aromatic, most preferably phenylalanyl, which has the L-configuration at the carbon alpha to the carbonyl group (C<sub>α</sub>), and preferably has a side chain of the formula 40 -CH<sub>2</sub>-Y wherein Y is selected from lower alkyl, phenyl, substituted phenyl and indole groups; and 40

Z is an amino blocking group) and the salts thereof.

The following abbreviations are used herein:

45 *Amino Acids* (all assumed to be L unless otherwise specified) 45

Phe	-	L-phenylalanyl group	
Cit	-	L-citrullyl group	
Ala	-	L-alanyl group	
50 Arg	-	arginyll group	50
Lys	-	lysyl group	
(NO <sub>2</sub> )Arg	-	N <sup>ω</sup> -nitroarginyll group	
(Ts)Arg	-	N <sup>ω</sup> -tosylarginyll (or N <sup>ω</sup> -p-toluene-sulfonyl arginyll) group	
55 Trp	-	tryptophyll group	55
Met	-	methionyll group	
Asp	-	aspartyll group	
(TFA)Lys	-	N <sup>ε</sup> -trifluoroacetyllysinyll group	
(CBZ)Lys	-	N <sup>ε</sup> -carbobenzylloxyllysinyll group	
60 Val	-	valyll group	60
Leu	-	leucyll group	
Ile	-	isoleucyll group	
Nala	-	Naphthylalanyl group	

*Amine Blocking Groups*

	CBZ	-	carbobenzyloxy group	
	Bz	-	benzoyl group	
5	t-BOC	-	t-butyloxycarbonyl group	5

*Chromophores and Fluorophores*

	AMC	-	7-amino-4-methylcoumarin group	
10	HMC	-	7-hydroxy-4-methylcoumarin group	10
	AFC	-	7-amino-4-trifluoromethylcoumarin group (See International Patent Application Publication No. WO 80/02295)	
	AQ	-	6-aminoquinoline group	
15	NA	-	2-naphthylamine group	15
	MNA	-	4-methoxy-2-naphthylamine group	
	PNP	-	para-nitrophenol group	
	PNA	-	para-nitroaniline group	

20	<i>Synthetic Reagents and Solvents</i>	20
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	TEA	-	Triethylamine	
	DCC	-	N,N'-dicyclohexylcarbodiimide	
	DMF	-	N,N-dimethylformamide	
25	THF	-	tetrahydrofuran	25
	DMSO	-	dimethylsulfoxide	
	HOAc	-	acetic acid	
	HBr/HOAc	-	acetic acid saturated with HBr	
	Pd-PEI	-	palladium polyethyleneimine catalyst	

30	<i>Miscellaneous</i>	30
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	EDTA	-	ethylenediaminetetraacetic acid, disodium salt	
35	mmole	-	millimoles	35
	mm	-	millimeter	
	nm	-	nanometer	
	$[\alpha]_D^{25}$ (DMF)	-	optical rotation at 589nm, 25°C, in DMF solvent	
40	Bis-Tris	-	2,2-bis(hydroxymethyl)-2,2',2''- nitrilotriethanol	40
	HPLC	-	high performance liquid chromatography using Alltech (Deerfield, Ill.) column: 4.6x250 mm, C18; flow rate: 1 ml/min; detector: UV 340, 313, 280, and 254 nm. Mobile phase solvent, retention time, and approximate purity listed for each example.	
45	MS	-	mass spectroscopy using a Micro Mass VG7070H instrument with fast atom bombardment probe (9KV Xenon) and sample dissolved in DMSO/glycerol.	45
50	H-NMR	-	proton nuclear magnetic resonance on a Bruker 270 MHz instrument using d <sub>6</sub> -DMSO solvent and tetramethylsilane as an internal standard at a chemical shift (δ) of 0.0. The chemical shifts, δ, the peak splitting: s = single, d = doublet, t = triplet, q = quartet, qn = quintet, se = sextet, c = complex multiplet, and the number of protons (1p = 1 proton) are listed for each example.	50
55				55
60	MP	-	melting point (uncorrected, Fisher-Johns apparatus)	60





5

for  $R_2$  is equal to Trp

- $\text{CH}_2\text{-C}_6\text{H}_5$  for  $R_2$  is equal to Phe
- $\text{CH}_2\text{-CH-(CH}_3)_2$  for  $R_2$  is equal to Leu
- $\text{CH-(CH}_3)_2$  for  $R_2$  is equal to Val

5

- 10 The identity of Z is less critical than that of  $R_1$  and  $R_2$ , but Z is useful in making small changes in affinity of the compound for cathepsin B. This Z group may be a simple amino protecting group, such as CBZ, BOC or Bz. Also, Z may be another blocked amino acid group, such as CBZ-(D or L configuration)-Ala. The Z group, in any event, should not interfere with the selective binding of cathepsin B to the  $R_1$  and  $R_2$  groups.

10

The synthesis of the peptide substrates can be effected by numerous generally accepted peptide synthetic methods. The following illustrates three synthetic strategies:

15 *Method A:* The group Z- $R_2$  is coupled to the  $R_1$ -X group by the DCC, mixed-anhydride or other typical coupling method.

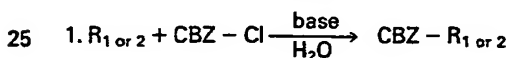
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*Method B:* The previously synthesized complete peptide Z- $R_2$ - $R_1$  is coupled with the indicator group X using the DCC, mixed-anhydride or other acceptable coupling method.

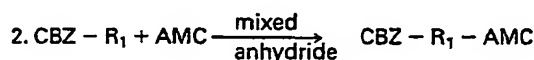
- 20 *Method C:* Sequential coupling of the groups Z,  $R_2$ ,  $R_1$  is followed by the coupling of the indicator group X using the DCC, mixed-anhydride or other acceptable coupling method.

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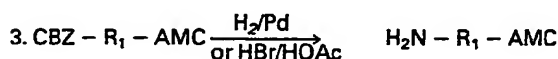
The general procedures for the majority of the compounds described in the examples are outlined below and follow the general *Method A*:



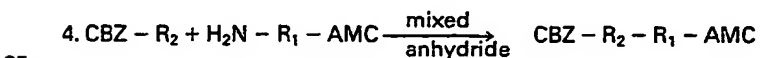
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(Where  $R_1$  and  $R_2$  denote different amino acid residues in the general formula, and CBZ is used for Z and AMC is used for X).

The methods and compounds of the invention will now be further illustrated by the following non limiting examples:

- 40 Mixed anhydride refers to the type of coupling procedure. The details of reaction 1-4 are described below.

40

(1) *CBZ-Cl Reaction:* In some cases the CBZ-R derivatives were commercially available, in others they were prepared by the following method: The amino acid R (25 mmole), and 50 mmole sodium bicarbonate were dissolved into 50 ml of water in a 100-ml flask. Carbobenzyloxy-chloride (27.5 mmole) was added with stirring at ambient temperature in 5 aliquots over one hour. After the solution was stirred for an additional two hours, it was extracted with ether twice and then dripped into 100 ml of 1M HCl. The product first precipitated as a semisolid and then solidified upon standing. The crude product was washed with water, dried and used without further purification. The average yield was 55%.

(2) *AMC - Coupling:* Into a 50-ml flask were added 11.4 mmole blocked  $R_1$ -amino acid, 15 ml anhydrous THF, and 11.54 mmole TEA. After the compound dissolved, the solution was cooled on ice, and 11.4 mmole of isobutylchloroformate was added. The solution was stirred for 10 minutes on ice, and then a chilled solution of 11.4 mmole AMC in 21 ml of DMF was added. Stirring was continued on ice for one hour then at ambient temperature overnight. The reaction mixture was filtered and the THF removed from the filtrate on a rotary evaporator. The residue was added to a 125-ml separatory funnel along with 20 ml  $\text{CH}_2\text{Cl}_2$  and 30 ml 10% aqueous HCl, and shaken vigorously. After extracting the organic phase with a second aqueous acid aliquot, the product precipitated in the organic phase. The aqueous phase was decanted and the precipitate collected by filtration and washed with ether. The product was dried and used without further purification. The average yield was 41%.

(3) *Deblocking:* The N-carbobenzyloxy blocking group was removed from Z- $R_1$ -AMC either by treatment with a hydrogen bromide/glacial acetic acid solution (HBr/HOAc), or catalytic hydrogenation. In two examples -t-BOC was used instead of CBZ, and deblocking was accomplished by formic acid treatment.

*HBr/HOAc:* Into a 500-ml flask were added 3.8 mmole of Z- $R_1$ -AMC and 70 ml of 33% HBr/HOAc. After the sample dissolved, the solution was stirred an additional 15 minutes. The reaction mixture was diluted with 500 ml ether and the resulting precipitate was collected by filtration under nitrogen. The product was resuspended three times in 100 ml ether and refiltered. The product was dried and used without further purification. The average yield was 98%.

65

**Catalytic Hydrogenation:** Into a 500-ml pressure flask were added 8.5 mmole Z-R<sub>1</sub>-AMC, 4 g of palladium-polyethyleneimine beads (Pd-PEI) and 200 ml of methanol. The flask was pressurized with 20 psig (0.138 MPa gauge) hydrogen and shaken for six hours, then allowed to stand for an additional 18 hours. After the catalyst beads settled, the alcohol was decanted and the beads were washed twice with 50 ml of methanol. To aid the purification, the hydrochloride salt was prepared by adding 12.5 ml of 1 N aqueous HCl. The solvent was removed under vacuum and the residue redissolved and reevaporated twice with 50 ml denatured alcohol. The residue was triturated with denatured alcohol and allowed to stand overnight. The product, R<sub>1</sub>-AMC was collected by filtration, washed with denatured alcohol, dried under vacuum, and used without further purification. The average yield was 78%.

**Formic Acid:** Into a 500-ml flask was added 1.75 mmole t-BOC-R<sub>1</sub>-AMC and 30 ml of 97% formic acid. After the compound dissolved, the solution was stirred at ambient temperature for 3 hours. To this was added 250 ml of ether and 0.2 ml of concentrated HCl (to form the HCl salt). The white precipitate was filtered and washed with ether. After drying under vacuum, the product was used without further purification.

**(4) Peptide Coupling:** Into a 50-ml flask were added 3.2 mmole of R<sub>1</sub>-AMC · HBr (or HCl), 25 ml anhydrous DMF and 3.2 mmole TEA. Into a second 50-ml flask were added 3.2 mmole CBZ-R<sub>2</sub>, 10 ml anhydrous DMF and 3.2 mmole TEA. After the compounds dissolved, both solutions were cooled on ice, and 3.2 mmole of isobutyl chloroformate was added to the CBZ-R<sub>2</sub> solution. This reaction mixture was stirred for 10 minutes on ice, and then the chilled R<sub>1</sub>-AMC solution was added to the CBZ-R<sub>2</sub> reaction mixture. Stirring was continued for one hour on ice and then overnight at room temperature. The reaction mixture was then dripped into 950 ml of 5% aqueous sodium bicarbonate with stirring. The precipitate was collected by filtration and washed three times with water. The average crude yield was 90%.

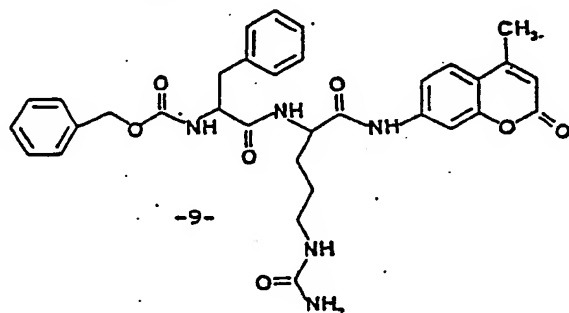
**Purification:** The final products were purified by the following crystallization and in some cases additional crystallizations and HPLC. The CBZ-R<sub>2</sub>-R<sub>1</sub>-AMC product (2.9 mmole) was dissolved into 35 ml of glacial acetic acid with stirring and gentle heating. To this was added 54 ml of acetonitrile and this solution was heated to reflux. The hot solution was filtered, and 80 ml of water was added to the filtrate in small aliquots with heating and stirring. A white precipitate formed upon cooling which was filtered and washed with water. The recrystallized product was dried under vacuum and the average crystallization yield was 80%. The overall yield and synthetic alterations are listed for each example below.

The chemical structures for the peptides discussed in this specification and appearing in the examples are given below:

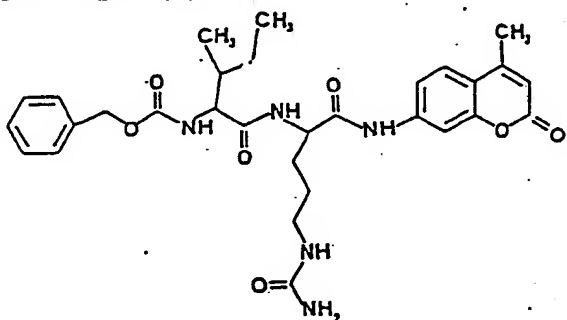
#### Example

#### No. Compound or Substrate

#### 1. CBZ - Phe - Cit - AMC



#### 2. CBZ - Leu - Cit - AMC



**.5**



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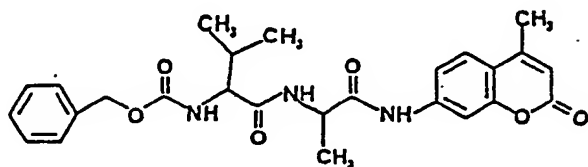


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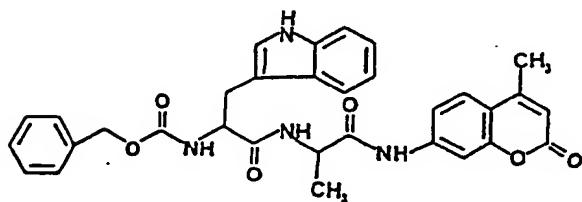
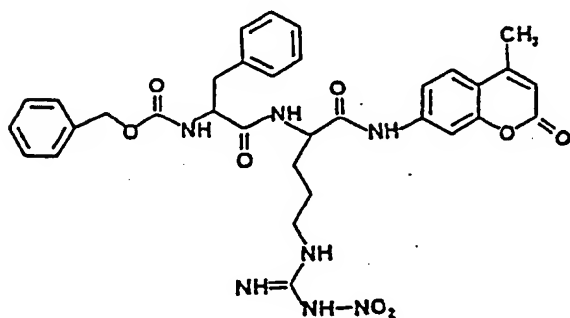




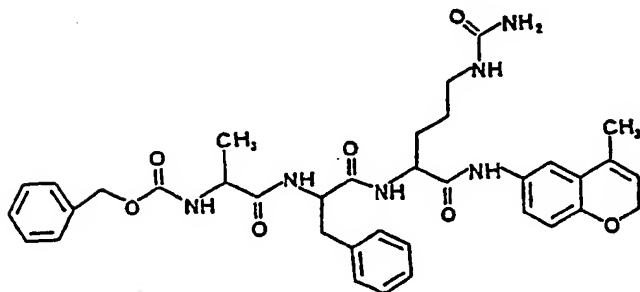
## 7. CBZ - Val - Ala - AMC



## 8. CBZ - Trp - Ala - AMC

9. CBZ - Phe - (NO<sub>2</sub>) Arg - AMC

## 10, 11. CBZ - (D or L) - Ala - Phe - Cit - AMC



## 12. CBZ - Phe - Met - AMC

5

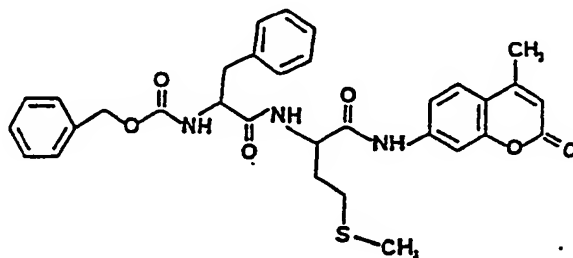
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## 13. CBZ - Phe - (Ts) Arg - AMC

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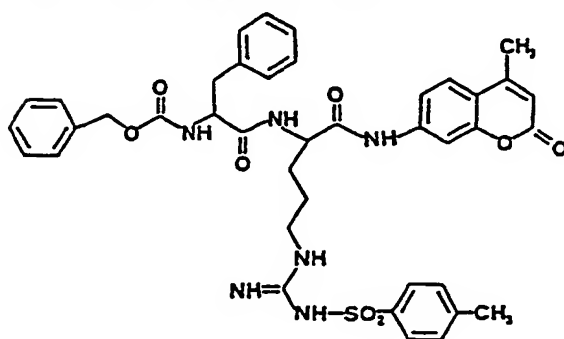
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## 14. CBZ - Phe - (CBZ) Lys - AMC

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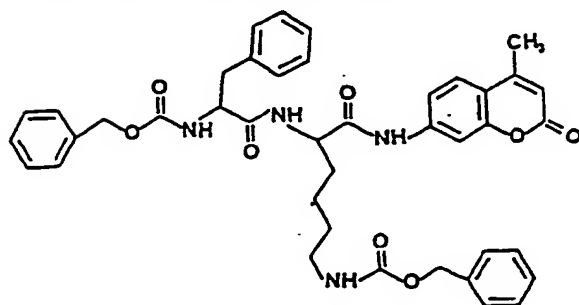
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## 15. CBZ - Phe - (TFA) Lys - AMC

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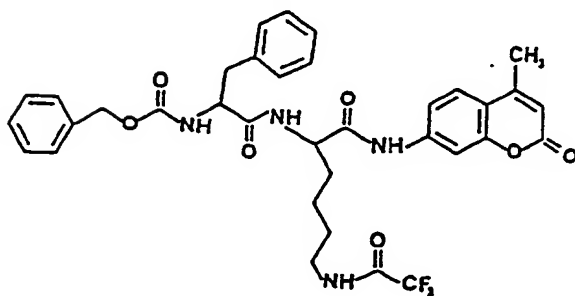
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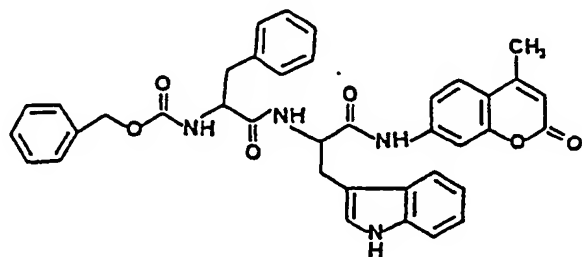
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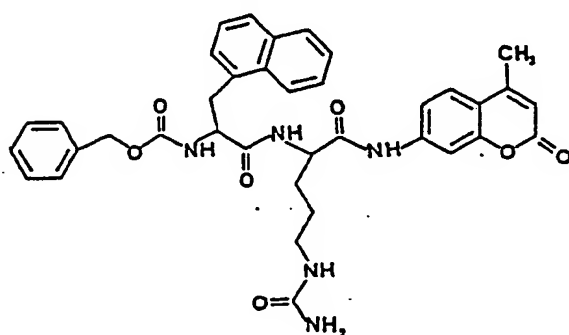
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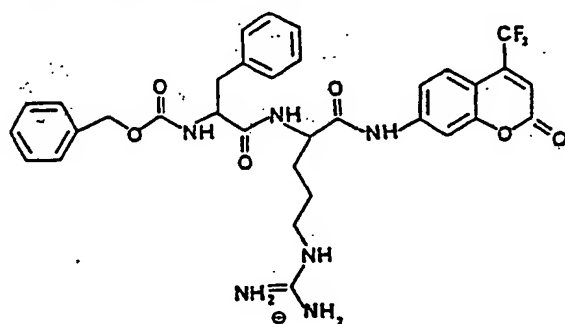
## 16. CBZ - Phe - Trp - AMC



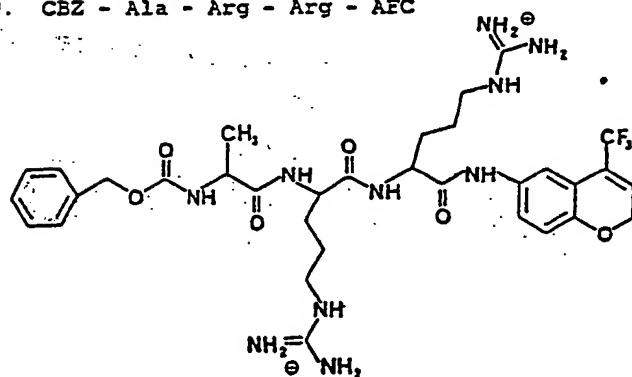
## 17. CBZ - Nala - Cit - AMC



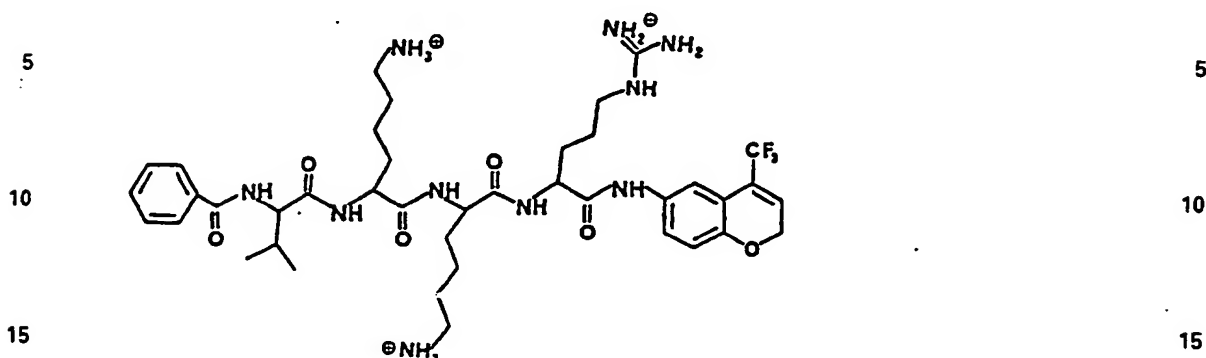
## 18. CBZ - Phe - Arg - AFC



## 19. CBZ - Ala - Arg - Arg - AFC



## 20. BZ - Val - Lys - Lys - Arg - AFC

*Substrate Synthesis*

*Example 1:* CBZ-L-Phe-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 17 ml HOAc, 74 ml CH<sub>3</sub>CN, 140 ml H<sub>2</sub>O per gram. The overall yield was 14%. *MP*=227-230°C.  $[\alpha]_D^{25}$  (DMF) = -22.77°. *HPLC* (70% CH<sub>3</sub>OH/30% H<sub>2</sub>O) 9.6 min, 97.3%. <sup>1</sup>*H-NMR*: δ 1.30-1.55, c, 2p; δ 1.55-1.81, c, 2p; δ 2.40, s, 3p; δ 2.75, t, 1p; δ 2.90-3.10, c, 3p; δ 4.28-4.40, c, 1p; δ 4.40-4.51, c, 1p; δ 4.94, s, 2p; δ 5.43, s, 2p; δ 6.00, t, 1p; δ 6.28, s, 1p; δ 7.10-7.39, c, 10p; δ 7.48, d, 1p; δ 7.51, d, 1p; δ 7.73, d, 1p; δ 7.80, d, 1p; δ 8.35, d, 1p; δ 10.51, s, 1p. *MS*: parent molecular ion = 614.

*Elemental Analysis:* calculated for C<sub>33</sub>H<sub>35</sub>N<sub>5</sub>O<sub>7</sub>, C 64.59%, H 5.75%, N 11.41%; found C 64.81%, H 5.77%, N 11.61%.

*Example 2:* CBZ-L-Leu-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 17 ml HOAc, 36 ml CH<sub>3</sub>CN, 136 ml H<sub>2</sub>O per gram. The overall yield was 19%. *MP*=196-198°C.  $[\alpha]_D^{25}$  (DMF) = -25.79°. *HPLC*, (45% CH<sub>3</sub>CN/55% H<sub>2</sub>O) 9.8 min, 99.9%. <sup>1</sup>*H-NMR*: δ 0.87, 2d, 6p; δ 1.44, t, 3p; δ 1.54-1.77, c, 4p; δ 2.39, s, 3p; δ 2.86-3.10, c, 2p; δ 4.10, q, 1p; δ 4.43, q, 1p; δ 5.04, s, 2p; δ 5.42, s, 2p; δ 5.98, t, 1p; δ 6.27, s, 1p; δ 7.24-7.53, c, 7p; δ 7.72, d, 1p; δ 7.78, d, 1p; δ 8.16, d, 1p; δ 10.46, s, 1p. *MS*: parent molecular ion = 580.

*Elemental Analysis:* calculated for C<sub>30</sub>H<sub>37</sub>N<sub>5</sub>O<sub>7</sub>, C 62.16%, H 6.43%, N 12.08%; found C 62.59%, H 6.50%, N 12.06%.

*Example 3:* CBZ-L-Ile-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 20 ml HOAc, 34 ml CH<sub>3</sub>CN, 54 ml H<sub>2</sub>O per gram. The overall yield was 19%. *MP*=240-242°C.  $[\alpha]_D^{25}$  (DMF) = -28.16°. *HPLC* (50% CH<sub>3</sub>CN/50% H<sub>2</sub>O) 7.1 min, 91.8%. <sup>1</sup>*H-NMR*: δ 0.73-0.93, c, 6p; δ 1.00-1.80, c, 7p; δ 2.40, s, 3p; δ 2.86-3.12, c, 2p; δ 3.96, t, 1p; δ 4.43, q, 1p; δ 5.05, s, 2p; δ 5.43, s, 2p; δ 5.99, t, 1p; δ 6.27, s, 1p; δ 7.25-7.42, c, 6p; δ 7.49, d, 1p; δ 7.72, d, 1p; δ 7.78, s, 1p; δ 8.20, d, 1p; δ 10.46, s, 1p. *MS*: parent molecular ion = 580.

*Elemental Analysis:* calculated for C<sub>30</sub>H<sub>37</sub>N<sub>5</sub>O<sub>7</sub>, C 62.16%, H 6.43%, N 12.08%; found C 62.24%, H 6.36%, N 12.14%.

*Example 4:* CBZ-L-Val-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 24 ml HOAc, 75 ml CH<sub>3</sub>CN, 128 ml H<sub>2</sub>O per gram. The overall yield was 16%. *MP*=244-247°C.  $[\alpha]_D^{25}$  (DMF) = -27.36°. *HPLC* (35% CH<sub>3</sub>CN/65% H<sub>2</sub>O) 17.1 min, 93.8%.

<sup>1</sup>*H-NMR*: δ 0.73-1.0, c, 6p; δ 1.30-1.54, c, 2p; δ 1.54-1.83, c, 2p; δ 1.83-2.10, c, 1p; δ 2.40, s, 3p; δ 2.83-3.12, c, 2p; δ 3.94, t, 1p; δ 4.43, q, 1p; δ 5.05, s, 2p; δ 5.42, s, 2p; δ 5.98, t, 1p; δ 6.28, s, 1p; δ 7.20-7.43, c, 5p; δ 7.44-7.49, c, 2p; δ 7.72, d, 1p; δ 7.77, d, 1p; δ 8.19, d, 1p; δ 10.46, s, 1p.

*MS*: parent molecular ion = 566.

*Elemental Analysis:* calculated for C<sub>29</sub>H<sub>35</sub>N<sub>5</sub>O<sub>7</sub>, C 61.58%, H 6.24%, N 12.38%; found C 61.86%, H 6.27%, N 12.44%.

*Example 5:* CBZ-L-Trp-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 16 ml HOAc, 37 ml CH<sub>3</sub>CN, 96 ml H<sub>2</sub>O per gram. The overall yield was 17%. *MP*=235-238°C.  $[\alpha]_D^{25}$  (DMF) = -23.75°. *HPLC*, (45% CH<sub>3</sub>CN/55% H<sub>2</sub>O) 5.2 min, 95.6%.

<sup>1</sup>*H-NMR*: δ 1.30-1.54, c, 2p; δ 1.54-1.83, c, 2p; δ 2.40, s, 3p; δ 2.82-3.40, c, 4p; δ 4.33-4.54, c, 2p; δ 4.96, s, 2p; δ 5.43, s, 2p; δ 5.99, t, 1p; δ 6.27, s, 1p; δ 6.95, t, 1p; δ 7.05, t, 1p; δ 7.10-7.45, c, 8p; δ 7.50, d, 1p; δ 7.65, d, 1p; δ 7.73, d, 1p; δ 7.80, d, 1p; δ 8.33, d, 1p; δ 10.49, s, 1p; δ 10.79, s, 1p.

*MS*: parent molecular ion = 653.

*Elemental Analysis:* calculated for  $C_{35}H_{36}N_6O_7$ , C 64.41%, H 5.56%, N 12.88%; found C 64.46%, H 5.48%, N 12.94%.

*Example 6:* CBZ-L-Phe-L-Ala-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 18 ml HOAc, 27 ml  $CH_3CN$ , 29 ml  $H_2O$  per gram. The overall yield was 31%.  $MP=232-234^\circ C$ .  $[\alpha]_D^{25}$  (DMF) =  $-23.41^\circ$ . *HPLC* (55% $CH_3CN/45\%H_2O$ ) 6.8 min, 98.2%.

$^1H-NMR$ :  $\delta$  1.36, d, 3p;  $\delta$  2.40, s, 3p;  $\delta$  2.74, t, 1p;  $\delta$  2.98-3.13, c, 1p;  $\delta$  4.33, c, 1p;  $\delta$  4.46, c, 1p;  $\delta$  4.94, s, 2p;  $\delta$  6.28, s, 1p;  $\delta$  7.12-7.40, c, 10p;  $\delta$  7.44-7.56, c, 2p;  $\delta$  7.74, d, 1p;  $\delta$  7.78, d, 1p;  $\delta$  8.40, d, 1p;  $\delta$  10.43, s, 1p.

*MS*: parent molecular ion = 528.

*Elemental Analysis:* calculated for  $C_{30}H_{29}N_3O_6$ , C 68.30%, H 5.54%, N 7.96%; found C 68.57%, H 5.68%, N 7.96%.

*Example 7:* CBZ-L-Val-L-Ala-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 100 ml HOAc, 104 ml  $CH_3CN$ , 67 ml  $H_2O$  per gram. The overall yield was 31%.  $MP=260-265^\circ C$ .  $[\alpha]_D^{25}$  (DMF) =  $-34.51^\circ$ . *HPLC* (45% $CH_3CN/55\%H_2O$ ) 6.8 min, 98.7%.

$^1H-NMR$ :  $\delta$  0.82-0.96, c, 6p;  $\delta$  1.33, d, 3p;  $\delta$  1.88-2.07, c, 1p;  $\delta$  2.40, s, 3p;  $\delta$  3.92, t, 1p;  $\delta$  4.44, c, 1p;  $\delta$  5.05, s, 2p;  $\delta$  6.28, s, 1p;  $\delta$  7.26-7.42, c, 5p;  $\delta$  7.45-7.52, c, 2p;  $\delta$  7.73, d, 1p;  $\delta$  7.76, d, 1p;  $\delta$  8.25, d, 1p;  $\delta$  10.39, s, 1p.

*MS*: parent molecular ion = 480.

*Elemental Analysis:* calculated for  $C_{26}H_{29}N_3O_6$ , C 65.12%, H 6.10%, N 8.76%; found C 65.59%, H 6.02%, N 8.79%.

*Example 8:* CBZ-L-Trp-L-Ala-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 33 ml HOAc, 47 ml  $CH_3CN$ , 44 ml  $H_2O$  per gram. The overall yield was 17%.  $MP=248-253^\circ C$ .  $[\alpha]_D^{25}$  (DMF) =  $-16.55^\circ$ . *HPLC* (45% $CH_3CN/55\%H_2O$ ) 13.8 min, 93.4%.

$^1H-NMR$ :  $\delta$  1.34, d, 3p;  $\delta$  2.40, s, 3p;  $\delta$  2.94, c, 1p;  $\delta$  3.08-3.22, c, 1p;  $\delta$  4.36, se, 1p;  $\delta$  4.45, t, 1p;  $\delta$  4.95, s, 2p;  $\delta$  6.26, s, 1p;  $\delta$  6.95, t, 1p;  $\delta$  7.05, t, 1p;  $\delta$  7.10-7.40, c, 8p;  $\delta$  7.50, d, 1p;  $\delta$  7.66, d, 1p;  $\delta$  7.72, d, 1p;  $\delta$  7.78, d, 1p;  $\delta$  8.36, d, 1p;  $\delta$  10.38, s, 1p;  $\delta$  10.79, s, 1p.

*MS*: parent molecular ion = 567.

*Elemental Analysis:* calculated for  $C_{32}H_{30}N_4O_6$ , C 67.83%, H 5.34%, N 9.89%; found C 68.00%, H 5.42%, N 9.96%.

*Example 9:* CBZ-L-Phe-L-(NO<sub>2</sub>)Arg-AMC was synthesized as detailed in steps 1-4 above using the HBr/HOAc deblocking in step 3, and recrystallization from 8 ml HOAc, 16 ml  $CH_3CN$ , 15 ml  $H_2O$  per gram. The overall yield was 20%.  $MP=150-160^\circ C$  (dec).  $[\alpha]_D^{25}$  (DMF) =  $-3.80^\circ$ . *HPLC* (55% $CH_3CN/45\%H_2O$ ) 8.8 min 95.9%.

$^1H-NMR$ :  $\delta$  1.4-1.9, c, 4p;  $\delta$  2.4, s, 3p;  $\delta$  2.7-3.1, c, 2p;  $\delta$  3.19, c, 2p;  $\delta$  4.34, c, 1p;  $\delta$  4.45, c, 1p;  $\delta$  4.95, s, 2p;  $\delta$  6.28, s, 1p;  $\delta$  7.10-7.42, c, 13p;  $\delta$  7.49, d, 2p;  $\delta$  7.74, d, 1p;  $\delta$  7.78, d, 1p;  $\delta$  8.34, d, 1p;  $\delta$  10.49, s, 1p.

*MS*: parent molecular ion = 658.

*Elemental Analysis:* calculated for  $C_{33}H_{35}N_4O_6$ , C 60.27%, H 5.36%, N 14.91%; found C 60.03%, H 5.41%, N 15.09%.

*Example 10:* CBZ-L-Ala-L-Phe-L-Cit-AMC was synthesized from the CBZ-L-Phe-L-Cit-AMC, (Example 1) by deblocking with HBr/HOAc as in step 3 above, followed by peptide coupling of that reaction product with CBZ-L-Ala as in step 4 above. The final product was recrystallized from 34 ml POAc, 44 ml  $CH_3CN$ , 273 ml  $H_2O$  per gram, and further purified for enzymatic analysis by *HPLC* (Prep., 45% $CH_3CN/55\%H_2O$ , 20 min). The overall crude yield was 40%.  $MP=236-240^\circ C$ .  $[\alpha]_D^{25}$  (DMF) =  $-39.91^\circ$ . *HPLC* (60% $CH_3CN/40\%H_2O$ ) 6.5 min, 92.1%.

$^1H-NMR$ :  $\delta$  1.14, d, 3p;  $\delta$  1.28-1.54, c, 2p;  $\delta$  1.54-1.83, c, 2p;  $\delta$  2.42, s, 3p;  $\delta$  2.73-3.14, c, 4p;  $\delta$  4.02, t, 1p;  $\delta$  4.44, q, 1p;  $\delta$  4.56, c, 1p;  $\delta$  5.00, q, 2p;  $\delta$  5.43, s, 2p;  $\delta$  5.99, t, 1p;  $\delta$  6.28, s, 1p;  $\delta$  7.08-7.40, c, 10p;  $\delta$  7.45, d, 1p;  $\delta$  7.53, d, 1p;  $\delta$  7.74, d, 1p;  $\delta$  7.79, d, 1p;  $\delta$  7.90, d, 1p;  $\delta$  8.28, d, 1p;  $\delta$  10.44, s, 1p.

*MS*: parent molecular ion = 685.

*Elemental Analysis:* calculated for  $C_{36}H_{40}N_6O_8$ , C 63.15%, H 5.89%, N 12.27%; found C 63.10%, H 5.91%, N 12.15%.

*Example 11:* CBZ-D-Ala-L-Phe-L-Cit-AMC was synthesized similar to Example 10 using CBZ-D-Ala and L-Phe-L-Cit-AMC in the final peptide coupling. The final product was recrystallized from 13 ml HOAc, 36 ml  $CH_3CN$ , 173 ml  $H_2O$  per gram, and further purified for enzymatic analysis by *HPLC* (Prep., 45% $CH_3CN/55\%H_2O$ , 20 min). The overall yield was 34%.  $MP=194-196^\circ C$ .  $[\alpha]_D^{25}$  (DMF) =  $-17.89^\circ$ . *HPLC*, (60% $CH_3CN/40\%H_2O$ ) 6.5 min, 88.4%.

$^1H-NMR$ :  $\delta$  0.98, d, 3p;  $\delta$  1.28-1.56, c, 2p;  $\delta$  1.56-1.88, c, 2p;  $\delta$  2.41, s, 3p;  $\delta$  2.78-3.20, c, 4p;  $\delta$  4.03, t, 1p;  $\delta$  4.44, c, 1p;  $\delta$  4.58, c, 1p;  $\delta$  4.95, q, 2p;  $\delta$  5.42, s, 2p;  $\delta$  6.00, t, 1p;  $\delta$  6.28, s, 1p;  $\delta$  7.10-7.38, c, 10p;  $\delta$  7.44, d, 1p;  $\delta$  7.54, d, 1p;  $\delta$  7.73, d, 1p;  $\delta$  7.80, d, 1p;  $\delta$  8.19, d, 1p;  $\delta$  8.24, d, 1p;  $\delta$  10.32, s, 1p.

*MS*: parent molecular ion = 685.

*Elemental Analysis:* calculated for  $C_{36}H_{40}N_6O_8$ , C 63.15% H 5.89%, N 12.27%; found C 62.97%, H 5.91%, N 12.20%.

*Example 12:* CBZ-L-Phe-L-Met-AMC was synthesized as detailed in steps 1-4 above using the HBr/HOAc deblocking in step 3 and recrystallization from 3 ml HOAc, 10 ml  $CH_3CN$ , 4 ml  $H_2O$  per gram, and further purified by HPLC (Prep., 60% $CH_3CN$ /40% $H_2O$ , 15 min) for enzymatic assay. The overall yield was 16%. *MP*=202-209°C.  $[\alpha]_D^{25}$  (DMF) = -3.79°. HPLC, (45% $CH_3CN$ /55% $H_2O$ ) 17.3 min, 85.2%.

$^1H$ -NMR:  $\delta$  1.85-2.20, c, 2p;  $\delta$  2.07, s, 3p;  $\delta$  2.35-2.62, c, 2p;  $\delta$  2.42, s, 3p;  $\delta$  2.76, t, 2p;  $\delta$  4.34, c, 1p;  $\delta$  4.53, c, 1p;  $\delta$  4.96, s, 2p;  $\delta$  6.28, s, 1p;  $\delta$  7.14-7.39, c, 10p;  $\delta$  7.50, d, 2p;  $\delta$  7.74, d, 1p;  $\delta$  7.78, d, 1p;  $\delta$  8.36, d, 1p;  $\delta$  10.48, s, 1p.

*MS:* parent molecular ion = 588.

*Elemental Analysis:* calculated for  $C_{32}H_{33}N_3O_6S$ , C 65.40% H 5.66%, N 7.15%, S 5.46, found C 65.76%, H 5.77%, N 7.09, S 5.45%.

*Example 13:* CBZ-L-Phe-L-(Ts)Arg-AMC was synthesized as detailed in steps 2-4 starting with the  $\alpha$ -t-BOC(Ts)Arg instead of the  $\alpha$ -CBZ derivative in step 2. The  $\alpha$ -t-BOC group was removed by formic acid as described above with a 17% yield. The overall crude yield was 9.2% and this material was further purified by HPLC (Prep., 75% $CH_3OH$ /25% $H_2O$ , 20 min). *MP*=118-125°C.  $[\alpha]_D^{25}$  + 7.0°. HPLC, (65% $CH_3CN$ /35% $H_2O$ ) 11.3 min, 90.9%.

$^1H$ -NMR:  $\delta$  1.29-1.58, c, 2p;  $\delta$  1.58-1.86, c, 2p;  $\delta$  2.28, s, 3p;  $\delta$  2.40, s, 3p;  $\delta$  2.74, t, 1p;  $\delta$  2.92-3.20, c, 3p;  $\delta$  4.24-4.52, d, 2p;  $\delta$  4.95, s, 2p;  $\delta$  6.28, s, 1p;  $\delta$  6.43-7.11, c, 3p;  $\delta$  7.11-7.44, c, 14p;  $\delta$  7.62, d, 2p;  $\delta$  7.75, d, 1p;  $\delta$  7.78, s, 1p;  $\delta$  8.34, c, 1p;  $\delta$  10.52, s, 1p.

*MS:* parent molecular ion = 767.

*Elemental Analysis:* calculated for  $C_{40}H_{42}N_6O_8S$ , C 62.65% H 5.52%, N 10.96%, S 4.18; found C 62.04%, H 5.66%, N 10.90, S 4.47%.

*Example 14:* CBZ-L-Phe-L-(CBZ)Lys-AMC was synthesized as detailed in steps 2-4 starting with the  $\alpha$ -t-BOC(CBZ)Lys instead of the  $\alpha$ -CBZ derivative in step 2. The  $\alpha$ -t-BOC group was removed by formic acid as described above with a 83% yield. Recrystallization was from 20 ml acetic acid, 56 ml  $CH_3CN$ , 127 ml  $H_2O$  per gram. The overall yield was 39%. *MP*=170-173°C.  $[\alpha]_D^{25}$  (DMF) = -4.28°. HPLC, (45% $CH_3CN$ /55% $H_2O$ ) 15.3 min, 97.5%.

$^1H$ -NMR:  $\delta$  1.20-1.54, c, 4p;  $\delta$  1.54-1.82, c, 2p;  $\delta$  2.40, s, 3p;  $\delta$  2.76, t, 2p;  $\delta$  2.88-3.09, c, 2p;  $\delta$  4.28-4.48, c, 2p;  $\delta$  4.96, d, 4p;  $\delta$  6.27, s, 1p;  $\delta$  7.10-7.43, c, 17p;  $\delta$  7.49, t, 1p;  $\delta$  7.73, d, 1p;  $\delta$  7.77, s, 1p;  $\delta$  8.29, d, 1p;  $\delta$  10.47, s, 1p.

*MS:* parent molecular ion = 719.

*Elemental Analysis:* calculated for  $C_{41}H_{42}N_4O_8$ , C 68.51% H 5.89%, N 7.79%; found C 68.23%, H 5.91%, N 7.69%.

*Example 15:* CBZ-L-Phe-L-(TFA)Lys-AMC was synthesized as detailed in steps 1-4 using the catalytic hydrogenation deblocking in step 3 and recrystallization from 28 ml  $CH_3CN$  per gram. The overall yield was 9%. *MP*=208-210°C.  $[\alpha]_D^{25}$  (DMF) = -8.15°. HPLC, (45% $CH_3CN$ /55% $H_2O$ ) 15.8 min, 80.4%.

$^1H$ -NMR:  $\delta$  1.20-1.45, c, 2p;  $\delta$  1.45-1.61, c, 2p;  $\delta$  1.61-1.85, c, 2p;  $\delta$  2.42, s, 3p;  $\delta$  2.75-3.06, c, 2p;  $\delta$  3.18, c, 2p;  $\delta$  4.28, se, 1p;  $\delta$  4.48, q, 1p;  $\delta$  4.95, s, 2p;  $\delta$  6.28, s, 1p;  $\delta$  7.14-7.39, c, 10p;  $\delta$  7.44-7.54, c, 2p;  $\delta$  7.74, d, 1p;  $\delta$  7.78, d, 1p;  $\delta$  8.31, d, 1p;  $\delta$  9.40, c, 1p;  $\delta$  10.48, s, 1p.

*MS:* parent molecular ion = 681.

*Elemental Analysis:* calculated for  $C_{35}H_{35}N_4O_7F_3$ , C 61.76% H 5.18%, N 8.23%, F 8.37%; found C 62.28%, H 5.38%, N 8.30, F 8.06%.

*Example 16:* CBZ-L-Phe-L-Trp-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3 and recrystallization from 11 ml HOAc, 11 ml  $CH_3CN$ , 4 ml and  $H_2O$  per gram. The overall yield was 31%. *MP*=165-167°C.  $[\alpha]_D^{25}$  (DMF) = +51.89°. HPLC, (45% $CH_3CN$ /55% $H_2O$ ) 16.2 min, 99.4%.

$^1H$ -NMR:  $\delta$  2.40, s, 3p;  $\delta$  2.74, t, 2p;  $\delta$  2.83-3.30, c, 2p;  $\delta$  4.33, qn, 1p;  $\delta$  4.76, q, 1p;  $\delta$  4.97, s, 2p;  $\delta$  6.28, s, 1p;  $\delta$  6.97, t, 1p;  $\delta$  7.07, t, 1p;  $\delta$  7.12-7.38, c, 13p;  $\delta$  7.48, d, 1p;  $\delta$  7.63, d, 1p;  $\delta$  7.72, d, 1p;  $\delta$  7.76, s, 1p;  $\delta$  8.38, d, 1p;  $\delta$  10.52, s, 1p;  $\delta$  10.86, s, 1p.

*MS:* parent molecular ion = 643.

*Elemental Analysis:* calculated for  $C_{38}H_{34}N_4O_6$ , C 71.01% H 5.33%, N 8.72%; found C 71.21%, H 5.40%, N 8.72%.

*Example 17:* CBZ-L-Nala-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3 and recrystallization from 40 ml HOAc, 86 ml  $CH_3CN$ , 92 ml and  $H_2O$  per gram. The overall yield was 13%. *MP*=225-227°C.  $[\alpha]_D^{25}$  (DMF) = -20.3°. HPLC, (60% $CH_3CN$ /40% $H_2O$ ) 8.0 min, 99.9%.

$^1H$ -NMR:  $\delta$  1.80-1.56, c, 2p;  $\delta$  1.56-1.85, c, 2p;  $\delta$  2.40, s, 3p;  $\delta$  2.84-3.22, c, 4p;  $\delta$  4.38-4.57, c, 2p;  $\delta$  4.91, s, 2p;  $\delta$  5.43, s, 2p;  $\delta$  6.00, t, 1p;  $\delta$  6.28, s, 1p;  $\delta$  7.07-7.65, c, 12p;  $\delta$  7.74, d, 1p;  $\delta$  7.78, d, 1p;  $\delta$  7.92, d, 1p;  $\delta$  8.22, d, 1p;  $\delta$  8.34, d, 1p;  $\delta$  10.50, s, 1p.

MS: parent molecular ion = 664.

Elemental Analysis: calculated for  $C_{37}H_{37}N_5O_7$ , C 64.59% H 5.75%, N 11.41%; found C 64.68%, H 5.87, N 11.40%.

## 5 Assay

The activity of cathepsin B is detected by monitoring the rate of release of indicator group X. The indicator group may be a moiety whose fluorescence or absorbance changes upon cleavage. Any of the known indicators such as AMC, HMC, AFC, AQ or PNA may be used. Alternatively, a radioactively labeled indicator may be employed.

- 10 The assay procedures for this enzyme, cathepsin B, follow the standard procedures and general principles adapted for enzymes (as described in A. J. Barrett, *op. cit.*; W. P. Jencks (1969) *Catalysis in Chemistry and Enzymology*, McGraw Hill, New York). A steady-state kinetic condition is set up using appropriate enzyme/substrate concentration ratios (the substrate concentration and its Michaelis constant greatly exceed the enzyme concentration) in order to obtain accurate rate information in the shortest laboratory time

15 scale.

The assay for cathepsin B activity is performed by adding a small quantity of a body fluid sample (1-50 microliters,  $\mu$ l) to an aqueous solution having a pH range at which cathepsin B is active, usually pH 5-6.5, preferably pH 5.5, containing EDTA and a thiol activator (biological antioxidant or other suitable reducing agent) such as cysteine or dithiothreitol. After a short activation period such as 1-2 minutes the substrate, dissolved in a compatible solvent such as methanol, ethanol, acetonitrile or DMF, is added in a small volume (20  $\mu$ l) such that the concentration of the substrate is substantially greater than the enzyme concentration (preferably at least 100 fold). The temperature is controlled to a set point, preferably in the range of ambient to about 37°C. After the sample and substrate are mixed, the concentration of cleaved indicator can be measured with respect to time in a spectrophotometer, spectrofluorometer or scintillation counter or other

25 known means which are well-known art and form no part of this invention.

A standard curve of known free indicator concentrations is developed so as to give a scale of comparison for unknown samples. The calculations would be as follows for a fluorescent indicator:

Equation 1:  $A \cdot B = C$

30 specifically,

$$(A \text{ fluorescence units/min}) (B) \frac{\mu\text{M indicator}}{\text{fluorescence units}} = (C \mu\text{M indicator/min.})$$

where A is obtained from the measurement with the sample, B is obtained from the standard curve, and C is the result. The term " $\mu$ M" refers to micromolar ( $\mu$ moles/liter), "ml" to milliliters, and min to minutes. Calculations would be made in a similar manner when other indicators are used. Information from this calculation provides a rate in terms of the concentration of the free indicator group X, and its change with time. This rate can be directly related to the rate of substrate cleavage by the enzyme, and thus, the amount of enzyme activity can be inferred.

## 40 Assay Examples

Purified cathepsin B was prepared from pig liver and neoplastic human liver using standard salting out procedures. The assay was performed using a 0.1 M Bis-Tris, acetate of phosphate buffer having pH 5.5 and containing 1mM EDTA. Cysteine (10mM) was used as the thiol activator, and the buffer containing the activator was freshly made each day. A small volume (5 $\mu$ l) of sample containing cathepsin B was mixed with 45 585  $\mu$ l of 37°C fresh buffer containing thiol activator in a 5x5 mm fluorimeter cuvette and incubated at 37°C for 2 minutes. Then 20 $\mu$ l of substrate in DMF at a concentration ranging from 0.05 mM to 3 mM was added with mixing. The increase in fluorescence was monitored with time at 37°C for several minutes using a Perkin Elmer LS-5 spectrofluorimeter. For AMC, the excitation wavelength was 350 nm, the emission wavelength 460 nm, and for AFC the excitation wavelength was 385 nm, the emission wavelength 490 nm. The fluorimeter settings used were: slit widths, 5 and 5mm, filter response, 1, and the scale factor was set at 0-1.0. The results of the enzymatic interaction with the substrate examples 1-17 and the commercial standards CBZ-Phe-Arg-AFC (Example 18), CBZ-Ala-Arg-Arg-AFC (Example 19) and Bz-Val-Lys-Lys-Arg-AFC (Example 20) are listed in Table 1.

TABLE 1

5	Ex No.	Compound	$V_{max}/K_m$		Rel. Order of Value <sup>3</sup>	5
			cathepsin B	Trypsin		
	1	CBZ-L-Phe-L-Cit-AMC <sup>1</sup>	0.296	NR <sup>2</sup>	2	
	2	CBZ-L-Leu-L-Cit-AMC	0.116	NR	6	
10	3	CBZ-L-Ile-L-Cit-AMC	0.126	NR	5	10
	4	CBZ-L-Val-L-Cit-AMC	0.169	NR	3	
	5	CBZ-L-Trp-L-Cit-AMC	0.148	NR	4	
	6	CBZ-L-Phe-L-Ala-AMC	0.034	NR	13	
	7	CBZ-L-Val-L-Ala-AMC	0.018	NR	14	
15	8	CBZ-L-Trp-L-Ala-AMC	0.069	NR	8	15
	9	CBZ-L-Phe-L-(NO <sub>2</sub> )Arg-AMC	1.025	NR	1	
	10	CBZ-L-Ala-L-Phe-L-Cit-AMC	0.105	NR	7	
	11	CBZ-D-Ala-L-Phe-L-Cit-AMC	0.062	NR	15	
	12	CBZ-L-Phe-L-Met-AMC <sup>4</sup>	0.614	NR	12	
20	13	CBZ-L-Phe-L-(Ts)Arg-AMC <sup>4</sup>	1.575	NR	9	20

1 AMC has a sensitivity of 0.049  $\mu$ M/fluorescence unit and was used as B in Equation 1 for these calculations.

2 NR - no reaction.

25 3 The order is based upon the rate of reaction with cathepsin B (the higher the rate the lower the order) and the rate of reaction with trypsin (the higher the rate the higher the order).

4 The relative value for this compound is reduced due to low solubility and low maximum rate.

TABLE 1 (continued)

30	Ex No.	Compound	$V_{max}/K_m$		Rel. Order of Value <sup>1</sup>	30
			cathepsin B	Trypsin		
35	14	CBZ-L-Phe-L-(Z)Lys-AMC <sup>4</sup>	0.182	NR	11	35
	15	CBZ-L-Phe-L-(TFA)Lys-AMC <sup>4</sup>	0.457	NR	10	
	16	CBZ-L-Phe-L-Trp-AMC	0.01	NR	17	
	17	CBZ-L-Nala-L-Cit-AMC	0.01	NR	16	
40	18	CBZ-Phe-Arg-AFC <sup>5</sup>	0.007	0.003	12	40
	19	CBZ-Ala-Arg-Arg-AFC <sup>5</sup>	0.003	0.058	14	
	20	BZ-Val-Lys-Lys-Arg-AFC <sup>5</sup>	0.017	0.077	13	

From these data it is clear that substrates 1-17 (Table 1) of the present invention have a much higher degree of selectivity and sensitivity for detecting cathepsin B in the presence of trypsin than the currently used peptides, such as the substrates 18-20 in Table 1 or similar peptides having a positively charged group at the R<sub>1</sub> position (attached to X). Since the tryptic enzymes are normally found in human blood, these enzymes interfere with cathepsin B measurement. The peptide substrates of the invention avoid or reduce the interference problems caused by trypsin-like enzymes, and increase the sensitivity for cathepsin B by 60-350 times that of the peptides of Examples 18-20.

In accordance with another embodiment of the invention, the activity of cathepsin B can be measured on a qualitative or semi-quantitative basis by contacting a prepared sample of the mammalian fluid with a suitable substrate of the invention. Typical clinical laboratory practices for obtaining such analyses might include, for example, cytology analysis in which the cell, tissue or organ sample is contacted with the substrate and other reagents for selective staining purposes. Still other typical clinical analyses include a test strip in which the substrate is incorporated into a suitable, relatively inert carrier which may be cellulosic or nonwoven material, or the combination thereof. When the sample is contacted with a test strip, the sample is absorbed into the test strip so as to result in a color change for a relatively quick analysis.

60 5 AFC has a sensitivity of 0.009  $\mu$ M/fluorescence unit and was used as B in Equation 1 for these calculations.

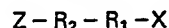
#### CLAIMS

65 1. A method of selectively assaying for the activity of cathepsin B in a material to be tested which may



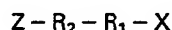
contain trypsin and trypsin-like enzymes, the steps comprising:

- (a) mixing a sample of said material with a substrate of formula I



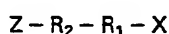
(I)

- 5 (wherein: 5
- X is an indicator moiety released by cleavage of the  $R_1 - X$  bond by cathepsin B and which is detectable upon cleavage;
- $R_1$  is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and which is not positively charged within the pH range for the assay; 10
- $R_2$  is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and
- Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the  $R_1$  and  $R_2$  groups) of an acid salt thereof,
- 15 said mixing being carried out in an aqueous medium having a pH within the range at which cathepsin B is active and using a quantity of said substrate substantially greater than that of cathepsin B and in sufficient concentration for cleaved X-groups to be detectable; and 15
- (b) measuring the rate of cleavage of the X-group from said substrate.
2. A method of detecting the activity of cathepsin B in a material to be tested which may contain trypsin and trypsin-like enzymes, and which is adapted to selectively assay for cathepsin B, the steps comprising: 20
- (a) contacting a sample of said material with a substrate of formula I



(I)

- 25 (wherein: 25
- X is an indicator moiety released by cleavage of the  $R_1 - X$  bond by cathepsin B and which is detectable upon cleavage;
- $R_1$  is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and which is not positively charged within the pH range for the detection method;
- 30  $R_2$  is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and 30
- Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the  $R_1$  and  $R_2$  groups) or an acid salt thereof,
- said contacting being carried out under conditions at which cathepsin B is active and at concentrations at which said substrate is present in amounts substantially greater than that of cathepsin B; and 35
- (b) determining the production of X cleaved from said substrate. 35
3. The method of either of claims 1 and 2 wherein X is a cleavable moiety whose fluorescence emission changes upon cleavage.
4. The method of any one of claims 1 to 3 wherein X is a cleavable moiety whose visible or ultraviolet spectrum changes upon cleavage. 40
5. The method of either of claims 1 and 2 wherein X is a 7-amino-4-methylcoumarin, 7-hydroxy-4-methylcoumarin, 7-amino-4-trifluoromethylcoumarin, 6-amino-quinoline, p-nitrophenol or p-nitroaniline group. 40
6. The method of any one of claims 1 to 5 wherein  $R_1$  is a citrullyl group.
- 45 7. The method of any one of claims 1 to 5 wherein  $R_1$  is a nitroarginyl group. 45
8. The method of any one of claims 1 to 7 wherein  $R_2$  is a phenylalanyl group.
9. The method of any one of claims 1 to 7 wherein  $R_2$  is a leucyl group.
10. The method of any one of claims 1 to 7 wherein  $R_2$  is a tryptophyl group.
11. The method of any one of claims 1 to 7 wherein  $R_2$  is an isoleucyl group.
- 50 12. The method of any one of claims 1 to 7 wherein  $R_2$  is a valyl group. 50
13. The method of any one of claims 1 to 5 wherein  $R_2$  is a phenylalanyl group and  $R_1$  is a citrullyl group.
14. The method of any one of claims 1 to 5 wherein  $R_2$  is a phenylalanyl group and  $R_1$  is a nitroarginyl group.
15. The method of any one of claims 1 to 5 wherein  $R_1$  is a citrullyl group and  $R_2$  is a valyl group.
- 55 16. The method of any one of claims 1 to 5 wherein  $R_1$  is a citrullyl group and  $R_2$  is a tryptophyl group. 55
17. The method of any one of claims 1 to 5 wherein  $R_1$  is a citrullyl group and  $R_2$  is an isoleucyl group.
18. The method of any one of claims 1 to 17 wherein Z is a carbobenzyloxy, benzoyl or t-butyloxycarbonyl group.
19. The method of any one of claims 1 to 18 wherein said material is a mammalian body fluid.
- 60 20. A method substantially as herein before described for determining cathepsin B activity in fluids using a compound of formula I (as defined in claim 1 or 2) or an acid salt thereof. 60
21. Compounds of formula I



(I)

(wherein:

X is an indicator moiety cleavable from R<sub>1</sub>;

R<sub>1</sub> is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group, and that is not positively charged;

5 R<sub>2</sub> is an hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

Z is an amino blocking group) and acid salts thereof.

22. The compounds of claim 21 wherein R<sub>1</sub> is a citrullyl group.

23. The compounds of claim 21 wherein R<sub>1</sub> is a nitroarginyl group.

10 24. The compounds of any one of claims 21 to 23 wherein R<sub>2</sub> is a phenylalanyl group. 10

25. The compounds of any one of claims 21 to 23 wherein R<sub>2</sub> is a leucyl group.

26. The compounds of any one of claims 21 to 23 wherein R<sub>2</sub> is a tryptophyl group.

27. The compounds of any one of claims 21 to 23 wherein R<sub>2</sub> is an isoleucyl group.

28. The compounds of any one of claims 21 to 23 wherein R<sub>2</sub> is a valyl group.

15 29. The compounds of claim 21 wherein R<sub>2</sub> is a phenylalanyl group and R<sub>1</sub> is a citrullyl group. 15

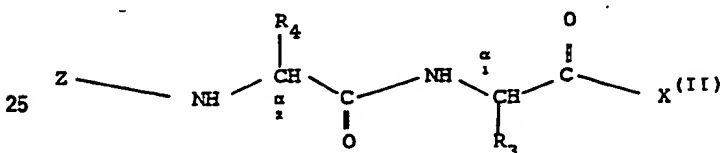
30. The compounds of claim 21 wherein R<sub>2</sub> is a phenylalanyl group and R<sub>1</sub> is a nitroarginyl group.

31. The compounds of claim 21 wherein R<sub>1</sub> is a citrullyl group and R<sub>2</sub> is a valyl group.

32. The compounds of claim 21 wherein R<sub>1</sub> is a citrullyl group and R<sub>2</sub> is a tryptophyl group.

33. The compounds of claim 21 wherein R<sub>1</sub> is a citrullyl group and R<sub>2</sub> is an isoleucyl group.

20 34. Compounds of formula II 20



(wherein:

30 X and Z are as defined in claim 21; 30

R<sub>3</sub> is the 2 to 8 atom containing side chain of an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group, and that is not positively charged; and

R<sub>4</sub> is the side chain of an hydrophobic amino acid residue which has the L-configuration at the carbon atom alpha to the carbonyl atom and R<sub>4</sub> has the following formula:

35  $-CH_2-Y$  35

wherein Y is selected from lower alkyl, phenyl, substituted phenyl and indole groups) and acid salts thereof.

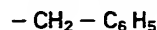
35. The compounds of claim 34 wherein R<sub>3</sub> is



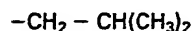
45 36. The compounds of claim 34 wherein R<sub>3</sub> is 45



37. The compounds of any one of claims 34 to 36 wherein R<sub>4</sub> is



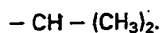
55 38. The compounds of any one of claims 34 to 36 wherein R<sub>4</sub> is 55



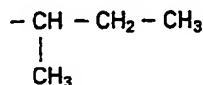
39. The compounds of any one of claims 34 to 36 wherein R<sub>4</sub> is

60  60

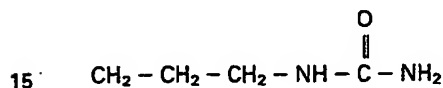
40. The compounds of any one of claims 34 to 46 wherein  $R_4$  is



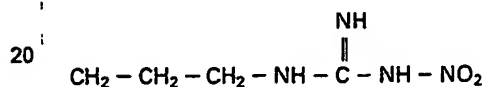
5 41. The compounds of any one of claims 34 to 36 wherein  $R_4$  is



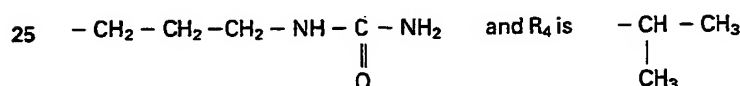
10 42. The compounds of claim 34 wherein  $R_4$  is  $-\text{CH}_2 - \text{C}_6\text{H}_5$  and  $R_3$  is



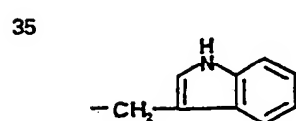
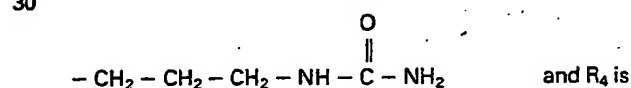
43. The compounds of claim 34 wherein  $R_4$  is  $-\text{CH}_2 - \text{C}_6\text{H}_5$  and  $R_3$  is



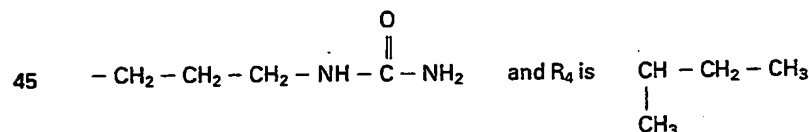
44. The compounds of claim 34 wherein  $R_3$  is



45. The compounds of claim 34 wherein  $R_3$  is



46. The compounds of claim 34 wherein  $R_3$  is



47. The compounds of any one of claims 21 to 46 wherein X is a cleavable moiety whose fluorescence changes upon cleavage.

48. The compounds of any one of claims 21 to 47 wherein X is a cleavable moiety whose visible or ultra-violet spectrum changes upon cleavage.

49. The compounds of any one of claims 21 to 48 wherein X is a 7-amino-4-methylcoumarin, 7-hydroxy-4-methylcoumarin, 7-amino-4-trifluoromethylcoumarin, 6-aminoquinoline, p-nitrophenol or p-nitroaniline group.

50. The compounds of any one of claims 21 to 49 wherein Z is a carbobenzyloxy, benzoyl or t-butyloxycarbonyl group.

51. Compounds as claimed in any one of claims 21 to 50 substantially as hereinbefore described in any one of Examples 1 to 17.

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